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Published by Associazione Teriologica Italiana
Hystrix, the Italian Journal of Mammalogy
Online first – 2019
OPEN ACCESS
Available online at:
http://www.italian-journal-of-mammalogy.it
doi:10.4404/hystrix-00128-2018

Research Article

Assessing a potential non-invasive method for viral diagnostic purposes in European squirrels

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Keywords:
SQPV
ADV
PCR
grey squirrels
red squirrels

Article history:
Received: 30 August 2018
Accepted: 29 May 2019

Acknowledgements
We thank Peter Trimming, Deb Edwards and other members of the public who reported and collected squirrel bodies for post mortem and analysis. We particularly thank Tony Dyda for providing grey squirrel carcasses from Gwynedd and Dr. Matt Hayward for provision of tissue storage at Bangor University. A special thank you goes to Nadia Inglese, Siva Karuna, Joanna Prince-Wright and Charlotte Bickers from Mammalian Virology, APHA for their help with elements of the PCR training and to the anonymous reviewers who have tirelessly helped turn the manuscript into a more focused article, suited for its intended audience. Finally, we acknowledge the LIFE14 NAT/UK/000467 Sciurious LIFE project which is integrating the novel PCR in ongoing applied invasive species management.

Abstract

Viral infections globally threaten wild and captive mammal populations, with surveillance options limited by a lack of non-invasive diagnostics; especially when infection is asymptomatic in nature. We explored the potential for hair samples collected from red (*Sciurus vulgaris*) and grey (*Sciurus carolinensis*) squirrels to provide a means of screening for adenovirus (ADV) and squirrelpox virus (SQPV) using evolving polymerase chain reaction (PCR) assays. An initial pilot study phase utilised samples opportunistically harvested from grey squirrels controlled in Gwynedd, United Kingdom (UK). The screening of 319 grey squirrel carcasses revealed 58% spleen ADV DNA qPCR and 69% SQPV antibody enzyme linked immunosorbent assay (ELISA) positives. We developed new nested ADV and SQPV qPCRs and examined tail hair samples from a sub-set of 80 of these 319 sampled squirrels and these assays amplified ADV and SQPV DNA in a higher proportion of animals than the original qPCR (94% and 21% respectively). Tail hair samples obtained from six Cumbrian red squirrels which had died from squirrelpox disease also revealed 100% SQPV and 50% ADV DNA positive by the nested qPCR assays. These findings indicate enhanced sensitivity for the new platform. The integration of this non-invasive approach in assessing viral infection has wide application in epidemiological studies of wild mammal populations, in particular, during conservation translocations, where asymptomatic infections are of concern.

Introduction

The spread of infectious disease in wild mammal populations can be elevated through climatic change, the international animal trade, conservation translocations and the spread of non-native species (Gavier-Widén et al., 2012). Where an infection can exist asymptomatically, it is currently often difficult to determine presence in wild populations. Trapping animals in order to examine them can elevate stress (Bosson et al., 2012). In addition, a particular infection may require invasive sampling of a nature that is difficult in field conditions (Ryser-Degiorgis, 2013). Here, we investigate the use of non-invasive hair sampling as a potential viral surveillance process, focussing on two infections in native and invasive European squirrels.

The introduced invasive eastern grey squirrel (*Sciurus carolinensis*) causes decline and regional extinction of the native Eurasian red squirrel (*Sciurus vulgaris*) in Europe (Gurnell et al., 2008, 2015; Romeo et al., 2018; Santicchia et al., 2018). It competes for resources and is an asymptomatic infection reservoir host for squirrelpox virus (SQPV) in the British Isles. Squirrelpox produces outbreaks of fatal epizootic disease in red squirrel populations (Rushton et al., 2006; Bruemmer et al., 2010; Chantrey et al., 2014). The infection is characterised typically by pustular lesions affecting the mouth, eyes, face, genitals and paws (McInnes et al., 2009). Transmission electron microscopy (TEM) is routinely used to confirm viral particle presence in lesion material taken from suspected infected red squirrels, while enzyme linked immunosorbent assay (ELISA) reveals an antibody presence from previous exposure in blood sera of both squirrel species.

Recently, pathogenic adenovirus (ADV) infection has been highlighted as a concern within wild red squirrel populations (Sainsbury et al., 2001; Duff et al., 2007; Martinez-Jiménez et al., 2011; Everest et al., 2010a, 2012a, 2014, 2017a). This infection produces enteric disease and can occur as an asymptomatic infection (Everest et al., 2014; Romeo et al., 2014). Viral particle presence in faecal material is associated with pathogenic infections and TEM is routinely used to confirm this in red squirrels (Everest et al., 2010b). In asymptomatic infections, blood is a poor medium, with spleen an optimal material (Shuttleworth et al., 2014). This presents a challenge when trying to determine asymptomatic infection, one particularly relevant to captive collections which provide stock for conservation trans-locations (Peters et al., 2011; Abendroth et al., 2017; Everest et al., 2017b, 2018). With a paucity of grey squirrel post mortem surveillance, currently, ADV can also only be described as being asymptomatic in nature in this species, with the importance of inter-specific infections currently unclear (see Everest et al., 2008; Dale et al., 2016 for published accounts).

SQPV infection in the grey squirrel is detected primarily as an asymptomatic presence (Everest et al., 2008; Dale et al., 2016), with

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low level tissue viral loads documented (Dale et al., 2016). Developing sensitivity enhanced assays for surveillance protocols would therefore be advantageous. Nested qPCR development allows positive detections where conventional qPCRs may not, offering definite analytical enhancements. Use of non-invasive sampling protocols for such studies has been limited (Wolf et al., 2004; Hall et al., 2010; Singh et al., 2011), but sourcing hair as a matrix for genetic studies has been more widely recorded (Hale et al., 2004; Ogden et al., 2005; O'Meara et al., 2018), but, limited to that function and not extended to utilise the material for viral studies. These platforms would however be ideally suited to examine cases where internal organs are not available for analysis, such as with bodies in an advanced state of decay, like those found in nest boxes, or pulverised following road traffic accidents where typically only skin may remain.

We investigated if infection presence could be detected in dead grey squirrel tail hair and muzzle whiskers previously found viral positive in spleen and blood. The same techniques were applied to dead red squirrel tail hair and muzzle whiskers for comparison. Evolution of a new PCR platform enabled comparison of the number of positive amplifications on material collected from the same individual animals relative to the older generation qPCRs used by Cowan et al. (2016). The effect of altering sample volume on PCR amplification rates were investigated, with results from both sciurid species presented and implications of the findings discussed in the context of non-invasive surveillance techniques.

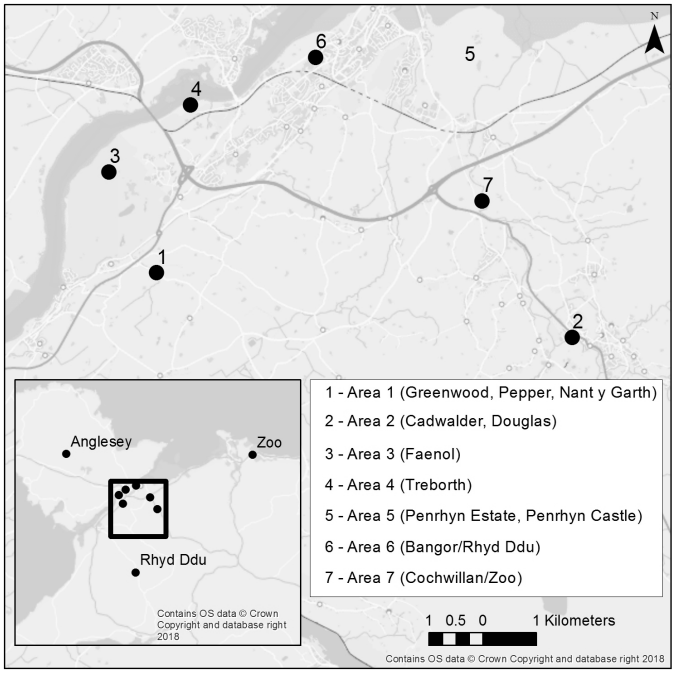

Figure 1 – Gwynedd grey squirrel woodland study area locations in relation to the island of Anglesey.

Materials and Methods

Sample collection

As a preliminary exercise to this study, grey squirrels were opportunistically obtained from a mainland control programme in Gwynedd, north Wales (see Cowan et al., 2016). These animals were culled between February and November 2014 with 319 carcasses obtained from seven woodland areas examined (Fig. 1). Following necropsy, blood, spleen, tail hair and muzzle whisker samples were submitted to the Animal and Plant Health Agency (APHA)-Weybridge for squirrel ADV PCR and SQPV ELISA analyses. ADV DNA was amplified in spleens from 186 animals. SQPV ELISA analyses were undertaken as part of the Cowan et al. (2016) study at the Moredun Research Institute. This current study utilised a randomly selected subset of 80 of these 186 spleen ADV and blood SQPV ELISA positive animals to examine hair and whiskers for both ADV and SQPV DNA presence by qPCR and nested qPCR assay platforms.

Red squirrel post mortem examination, histopathological findings and cause of death

Archived muzzle whisker samples from 51 dead red squirrels originating from Wales, England, Scotland and the grey squirrel-free Channel Island of Jersey (from 2015 and 2016) were obtained. Samples were received with varying necropsy information, with only 33 (65%) having a pathology report indicating cause of death. None had data relating to its ADV infection status, or signs associated with pathogenic SQPV. Mortality was attributable to a diverse range of infectious and non-infectious conditions with 18/33 (55%) due to road traffic collisions (Tab. S1).

DNA isolation and PCR methodology

The Kingfisher robotic system, protocol NM-LSI_RRC96 and LSI MagVet Universal Isolation Kit (Thermo Fisher Scientific, UK) were used for DNA extraction according to the manufacturer's instructions and DNA extracts were measured using a NanoDrop^{TM} 2000 spectrophotometer (ThermoFisher Scientific) to ensure process success. All sample nucleic acid and reagent dispensing was undertaken within MSC Class II or PCR workstations. All samples were tested in duplicate in the PCR and each DNA isolation and PCR run contained a negative extraction control (NEC), no-template control (NTC) and positive control. The NEC and NTC were to ensure lack of cross-contamination and set the background fluorescence in the qPCR. The positive control was to ensure optimal PCR performance between runs.

Primers and probes were designed using AlleleID Software (PREMIER Biosoft International, USA) and sequences initially verified using the BLAST search to ensure specificity (Tab. S2). Inner and outer primer testing was undertaken in a SYBR Green real time PCR assay using 10 μl Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, UK), 0.1 μl of each forward and reverse primers (100 pmol/μl), 2 μl of template DNA and water to a volume of 20 μl. Cycling conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 5 sec and 60 °C for 10 sec and terminated by melting curve analysis from 65 °C to 95 °C at an increment of 0.5 °C and continuous fluorescent measurement.

ADV qPCR

The Hexon gene of squirrel ADV (accession number KY427939.1) was used to design squirrel ADV qPCR primers and probe. The PCR mix was prepared in a clean room by adding 7.375 μl of PCR grade water to 2.5 μl 5x QuantiFast Pathogen PCR Master Mix (Qiagen). Primer sets and probe volumes were standardised at 100 pmol/μl and 0.05 μl of each forward 5'-CTCACTCCTAACGAATTC-3' and reverse 5'-CAGTCTTTTGTCATGTTAC-3' primers and 0.025 μl of probe FAM-CACATTGTATCCTTCTCCATCG-BHQ1 added to the mix. 10 μl of this mix was added to each PCR plate well and finally, 2.5 μl of sample nucleic acid added to each well, but inside a PCR workstation in a separate room. Plates were sealed and placed into a CFX96 Touch^{TM} Real-Time PCR (Bio- Rad^{TM}) for amplification, utilising 1 cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 sec, then 57 °C for 30 sec.

ADV nested qPCR

The ADV nested qPCR was performed using a 12.5 μl total volume, including 2.5 μl of extracted DNA. The remaining mixture contained 7.3 μl of RNase- free water, 2.5 μl of 5x QuantiFast Pathogen PCR Master Mix, 0.05 μl of the each forward and reverse primers and 0.025 μl of the probe. The nested qPCR was conducted on the CFX96 Touch^{TM} Real-Time PCR at 95 °C for 5 min, then 15 cycles of 95 °C for 15 sec, 68 °C for 30 sec followed by 40 cycles of 95 °C for 15 sec and 57 °C for 30 sec. The outer primers were, forward: 5'-GTG AGT TGG CCT GGC AAT GAT AGA C-3' and reverse: 5'-CGC GGT ACC

ATA GCT GCT AAA TCC-3' and inner primers and probe were as described for the ADV qPCR above.

SQPV SYBR Green real time PCR

The SYBR Green real time SQPV PCR primers were as described (Atkin et al., 2010). The PCR consisted of 12.5 μl Brilliant III Ultra-Fast SYBR®Green QPCR Master Mix (Agilent Technologies), 0.1 μl of each forward and reverse primers (100 pmol/μl), 10.3 μl water and 2 μl of sample nucleic acid. The PCR was run at 95 °C for 15 min followed by 40 cycles of 95 °C for 30 sec, 68 °C for 30 sec, 72 °C for 1 min and a final extension of 72 °C for 5 min. A melt curve analysis was performed from 65 °C to 95 °C at an increment of 0.5 °C on all samples immediately after the final extension stage.

SQPV nested qPCR

The DNA polymerase catalytic subunit gene of SQPV (accession number NC_022563.1) was used to design the SQPV nested qPCR primers and probe. The assay was performed as outlined for squirrel ADV, but using SQPV specific primers and probe. Outer primers were, forward: 5'-GAG CGC GAC ACC AGC GAG TTC AG-3' reverse: 5'-GAG CGT CTC CAA CTC CGC CTT CCT-3', and inner primers, forward: 5'- GTC AGC ATC AGG TAC ATG -3' reverse: 5'- ACA CCT ACT TTT CCA ACA -3' and probe: FAM-ACC GAG GTC CAC GAG ATC AG-BHQ1.

Grey squirrel samples and qPCR testing

In a preliminary investigation (Cowan et al., 2016), we selected whiskers (five/animal) from the known 11 ADV spleen positive animals from one area (Area 3), testing them for ADV and SQPV using standard (non-nested) qPCR assays. In addition, hair samples (5, 10 or 30 hairs) were obtained and similarly processed. Finally, whisker samples from 185 of the 186 spleen ADV positive animals were tested using the qPCRs.

In this study, we tested both five and 30 whole tail hairs with intact root bulbs from each of the 11 area 3 animals using the qPCR respectively. Finally, we selected five whiskers/sample for ADV qPCR analyses from each of 35 ADV DNA spleen negative grey squirrels, meaning we had data on relative whisker, hair and spleen for comparison.

Grey squirrel samples and nested qPCR testing

We randomly selected 80 from the 185 spleen ADV positive animals previously tested by the qPCR, and re-assayed the stored whisker DNA extracts from these 80 animals with the new nested qPCR. Comparing whisker and hair as test source materials, we assayed 30 whole hairs containing root bulbs using the nested qPCR. The same hair DNA extracts were used to perform the SQPV nested qPCR. We then applied ADV and SQPV nested qPCRs to 10 and then 30 whole hairs/sample from a further 24 randomly selected ADV and SQPV qPCR negative grey squirrels.

Red squirrel samples, qPCR and nested qPCR testing

We examined whole tail hairs (30 hairs/animal) for ADV from all 51 red squirrels opportunistically collected during national mortality surveillance by qPCR. In addition, whole hair samples from six TEM confirmed SQPV animals from Cumbria were tested for ADV and SQPV using nested qPCRs.

Results

qPCR and nested qPCR assays

The squirrel ADV qPCR developed in this study reliably detects down to 7.2 DNA copies using a serial dilution of a synthetic DNA template (Tab. S2). The qPCR was then trialled using two outer primer sets, with DNA extracted from four squirrel ADV positive samples. The best performing primer pair was selected based on the Ct values for use in the nested qPCR.

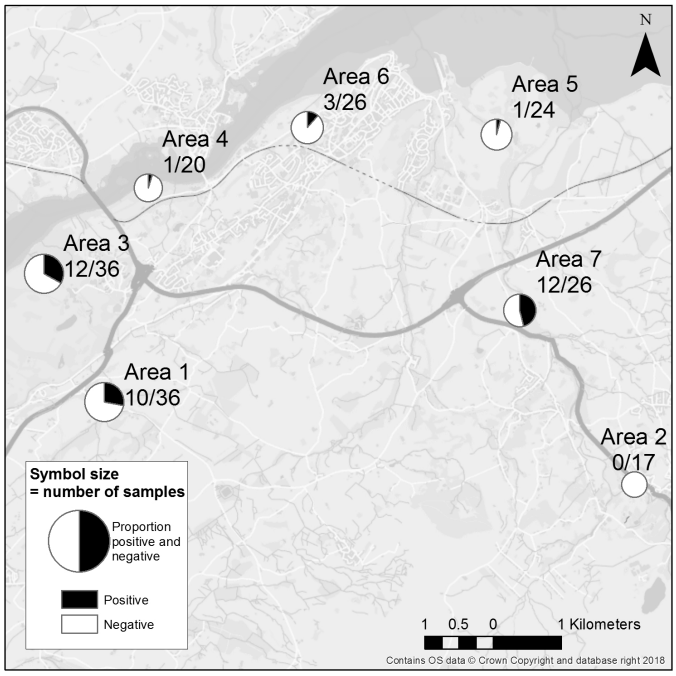

Figure 2 – Locations for 185 ADV qPCR grey squirrel whisker results (Rhyd Ddu and Zoo locations not displayed but data included).

To establish the nested SQPV qPCR, the late transcription factor gene (accession number HE601899.1) was used initially to design three primer pair sets. These sets were first tested using six known SQPV positive samples in the SYBR Green PCR assay. One primer set was chosen based on the Ct values and lack of primer dimers, however, this set in combination with the probe failed to perform optimally in the qPCR. Further primer sets were designed on the catalytic subunit of DNA polymerase gene and a 10- fold dilution series of a SQPV positive skin sample was used to assess their performance in a SYBR Green assay. One set of primers which lacked primer dimers and proved the most sensitive was selected. Optimum annealing temperature for the primers/probe combination was determined using four SQPV positive samples from 55 °C to 59.5 °C and an annealing temperature of 57 °C chosen. The primer/probes were further assessed using the four SQPV positive samples annealing at 57 °C with reliable amplification for each sample replicate. Finally, four outer primer combinations were tested in the qPCR using the four SQPV positive samples. The two most sensitive outer primer pairs were further tested in the nested qPCR using serial dilutions of a positive SQPV sample to select the appropriate primer pair.

Testing whisker and hair matrices using ADV and SQPV qPCR and nested qPCR

Using the qPCR, ADV DNA was amplified from whiskers from 12/36 (33%) grey squirrels known to be spleen ADV qPCR positive. No whisker samples were SQPV SYBR-Green real time PCR positive. A subsequent ADV qPCR examination of 185 spleen positive grey squirrels using five whiskers/sample revealed 39/185 (21%) positive (Fig. 2). Whisker samples from 35 ADV negative grey squirrel spleens, (five randomly selected from each woodland area), were analysed using the ADV qPCR, 3/35 (9%) positive.

Tail hair root bulb samples from 11 spleen ADV qPCR and SQPV antibody positive grey squirrels (identified in Cowan et al., 2016) were analysed using the ADV qPCR and SQPV SYBR-Green real time PCR assays. All were ADV and SQPV negative with five hairs/sample, but at 30 hairs/sample, 6/11 (55%) were ADV DNA and 8/11 (73%) SQPV DNA positive. Sequence analysis of these eight SQPV positives showed a 100% homology in each case to the published SQPV sequence (GenBank Accession number NC_022563.1), confirming SQPV DNA in root bulb material.

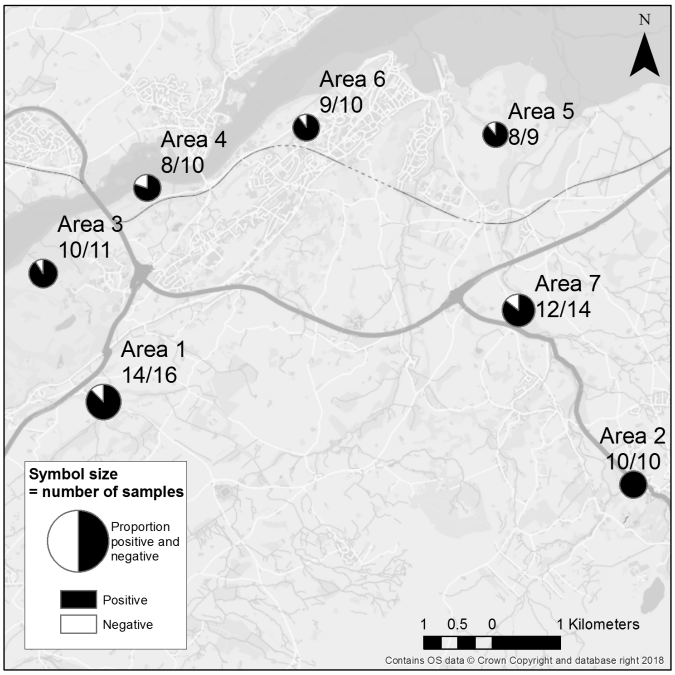

Figure 3 – Locations for 80 selected ADV nested qPCR grey squirrel whisker results (Rhyd Ddu and Zoo locations not displayed but data included).

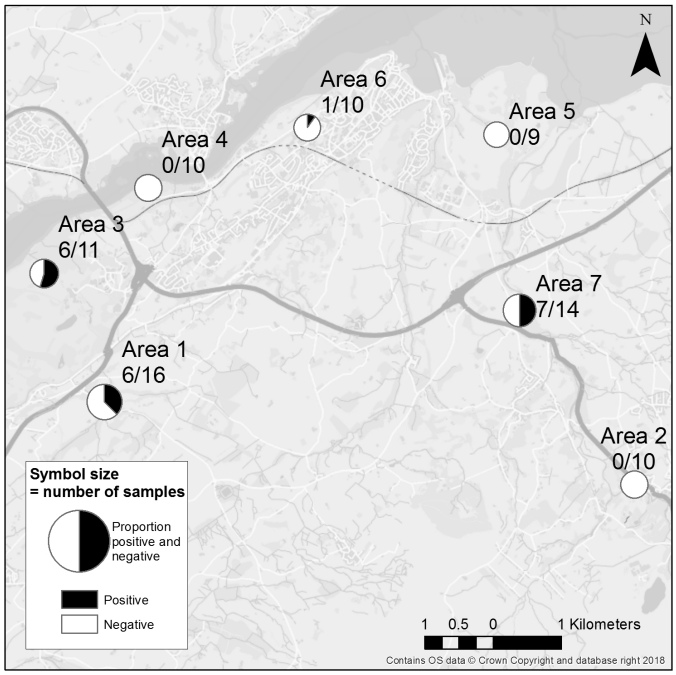

Figure 4 – Locations for 80 selected ADV qPCR grey squirrel whisker results (Rhyd Ddu and Zoo locations not displayed but data included).

ADV nested qPCR analyses on the archived whisker DNA extracts from the randomly selected 80 ADV qPCR positive spleens detected 71/80 (89%) ADV positive (Tab. 1, Fig. 3). This compared to 20/80 (25%) whisker positives using the qPCR (Tab. 1, Fig. 4), illustrating the enhanced sensitivity of the new PCR platform. A comparison using 30 whole hairs/sample instead of whisker with the new ADV nested qPCR, revealed an even higher number of ADV DNA positive samples, 75/80 (94%) (Tab. 1, Fig. 5). Direct comparison (two-sided Fisher's exact test) of the ADV qPCR against the ADV nested qPCR platform on whisker samples from these 80 animals revealed 20 and 71 ADV positives respectively, a difference that was statistically significant (p<0.0001). Comparison of the ADV nested qPCR platform on both whisker and hair samples from the same 80 animals revealed 71 and 75 ADV positives respectively and no statistical significance (p=0.4022). Using the same 80 hair DNA extracts (30 hairs/sample), SQPV nested qPCR analyses detected 17/80 (21%) positives (Tab. 1, Fig. 6). For the 35 selected ADV spleen negative animals, 3/35 (9%) were positive using the ADV qPCR. ADV and SQPV nested qPCR use with 10 whole hairs/sample from a further 24 randomly selected ADV and SQPV negative grey squirrels from the Cowan et al. (2016) study gave 19/24 (79%) ADV (Fig. 7), but no SQPV positives. SQPV nested qPCR analyses at 30 hairs/sample also detected no positives.

Of the 51 red squirrels analysed by the ADV qPCR only, 20/51 (39%) were ADV DNA whisker root bulb positive (Tab. 2), ranging from 69% on Anglesey to (20%) from Jersey. Whole tail hair samples from the additional six Cumbria SQPV outbreak animals showed 3/6 (50%) ADV positive and all six SQPV DNA positive respectively using the nested qPCR assays (Tab. 2).

Table 1 – ADV qPCR and ADV and SQPV nested qPCR results for 80 matched grey squirrel hair and whisker samples.

	qPCR		Nested qPCR	
Pathogen	Whisker	Hair	Whisker	Hair
ADV	20/80 (25%)	-	71/80 (89%)	75/80 (94%)
SQPV	-	-	-	17/80 (21%)

Table 2 – ADV and SQPV qPCR and nested qPCR results of hair and whisker samples from 57 dead wild red squirrels .

Location Tested	No. Positive	No. Tested	% Positive
Anglesey whisker			
ADV qPCR	11	16	69%
Scotland whisker			
ADV qPCR	3	10	30%
Jersey whisker			
ADV qPCR	4	20	20%
Cumbria whisker			
ADV qPCR	2	5	40%
Cumbria Hair			
ADV nested qPCR	3	6	50%
Cumbria Hair			
SQPV nested qPCR	6	6	100%

Discussion

Our findings demonstrate the possibilities for hair analysis as a non-invasive platform to determine viral infection status of native and introduced sciurids in Europe. This finding has obvious potential for transferability to infection monitoring of other mammalian species and is an exciting advancement. Having proven the concept with muzzle whiskers and demonstrated the technique's effectiveness with tail hair, the research has enhanced testing possibilities of tail/body hair collected directly from live-trapped animals or obtained from the environment. For example, hair tube or feeding station use to collect mammalian hair is routinely used in genetic studies (Finnegan et al., 2007; Sheehy et al., 2018) and is an optimal collection system to determine viral presence within a population in any given area. However, where different individuals deposit hair at the same point location, it may not be suited for individual animal studies.

We arbitrarily chose five whiskers and 10 hairs/sample to ensure sufficient source material availability and demonstrated further detections would result with additional matrix use at 30 hairs/sample. This is a similar description that Singh et al. (2011) presented in their study involving bovine viral diarrhoea virus vaccinated animals. The increase in hair numbers, from 10 to 20, BVDV positive cases increased, until using 30+ hairs/sample, where all cases were positive, correlating PCR results with matched blood samples. In addition, hair root bulbs and

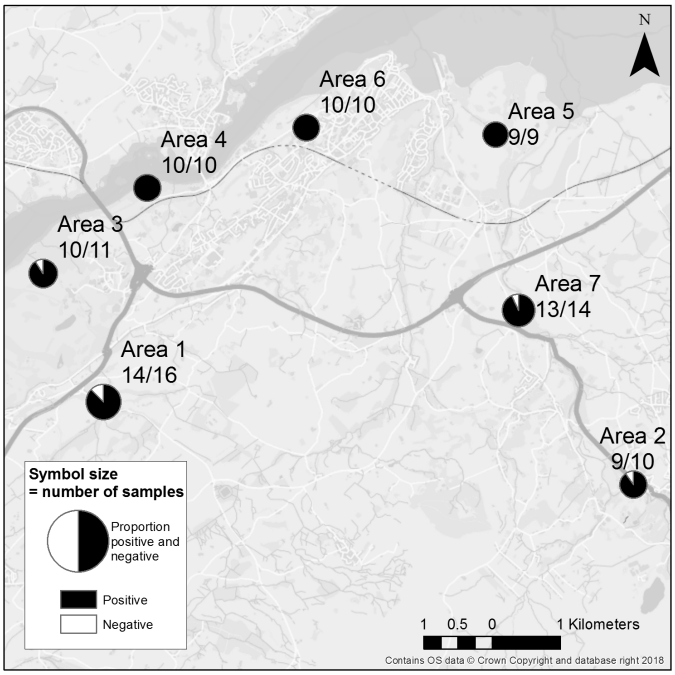

Figure 5 – Locations for 80 corresponding ADV nested qPCR grey squirrel hair results (Rhyd Ddu and Zoo locations not displayed but data included).

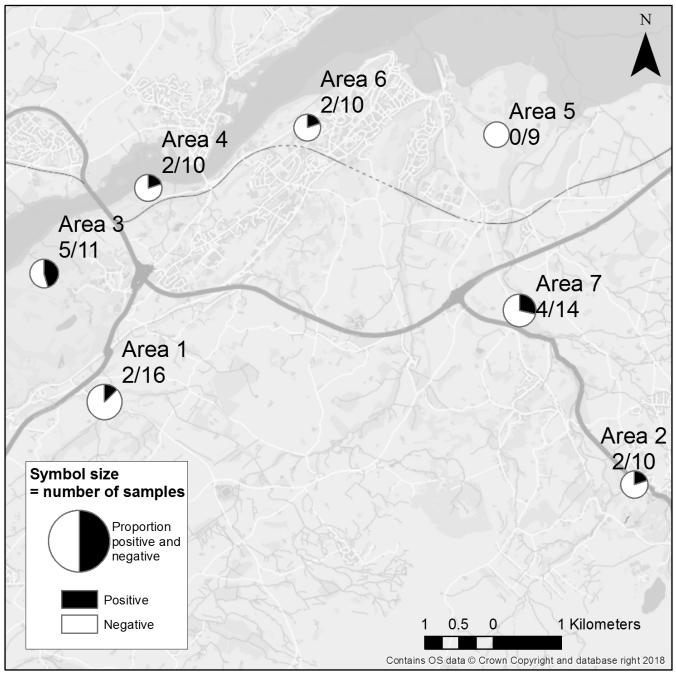

Figure 6 – Locations for 80 corresponding SQPV nested qPCR grey squirrel hair results (Rhyd Ddu and Zoo locations not displayed but data included).

shafts gave similar results (data not described) when using the same numbers from the same animals, allowing whole hair use for ease of operation. However, when we utilised 50 hairs/sample, positives were detected, but assay inhibition also encountered, with reduced detection levels. This highlights the importance of internal controls, spike controls or housekeeping genes such as beta actin, to check for PCR inhibition.

The results obtained clearly show that the nested qPCR shows greater sensitivity for the two viruses tested in the designed format than the established qPCR platform. We do not claim nor present these findings as a fully validated test procedure, but to indicate that this may be a possibility with further validation. The established qPCR presented 21% ADV positive whisker samples from the previously tested spleen positive animals, but using the same extracts, the nested qPCR format detected 89% ADV positive. Similarly, when using hairs at 30 hairs/sample, the nested assays detected 94% ADV positive from the same animals and in addition, 21% positive for SQPV DNA. Resource constraints precluded us from re-assaying the original spleen nucleic acid sample extracts, but, we are confident that we would have a similarly elevated level of ADV positive spleen results for proper technology comparison.

The level of ADV detected in this study was unexpected. ADV is generally regarded as being an infection of the haemolymphatic and gastrointestinal system and not topical as with SQPV. These results may well reflect ADV presence on hair as an environmental contaminant, but that is acceptable in the context of this study. The study offers further wildlife surveillance opportunities, as it identifies animals having been in an environment with the virus present and that other animals could potentially acquire the virus. Our findings might be extrapolated to other species deemed suitable for trans-location such as the hazel dormouse, or even pine marten (*Martes martes*), which prey on small rodent species carrying potentially a range of pathogenic infections (Tab. S3). Indeed, Walker et al. (2017) report on a number of novel adenovirus detections in British mustelids including pine marten. Therefore, pre- and post-movement viral analyses, should be considered essential deployment tools during future trans-locations.

In a combined qPCR and nested qPCR approach, lip tissue was examined from all 80 selected hair sampled animals with 1% positive for SQPV DNA, providing similar results to the Collins et al. (2014) and Shuttleworth et al. (2014) studies. Using a two-sided Fisher's exact test, directly comparing our 21% SQPV DNA nested qPCR hair detections against the tissue result described above, produces a statistically significant value (p=0.0001) favouring the hair platform. However, Chantrey et al. (2014) present a 27% detection rate, comparable to our 21% SQPV DNA nested qPCR hair detections, providing a potential additional matrix for examination.

Collins et al. (2014) and Shuttleworth et al. (2014) reported grey squirrel ELISA SQPV antibody levels of 22% and 33% and using skin or lip tissue, report 8% and 0% by PCR. Dale and Chantrey (2015) recorded tissue SQPV PCR levels of 9%. The Cowan et al. (2016) overall study value of 69% for SQPV antibody presence compares favourably, but our small-scale qPCR result of 73% SQPV DNA hair root bulb positives, combined with our nested qPCR result of 21% using 30 whole hairs/sample (Fig. 6), overall gave 28% SQPV positives, exceeding these reported values, indicating potential to provide a more realistic analytical matrix. We also detected 55% as ADV DNA positive. In our experience, pathological changes due to ADV are not apparent in grey squirrels, nor evidence of enteric abnormality in live animals, or carcasses on gross examination (Everest et al., 2009b). No TEM ADV particle detection has been recorded in the species, but Cowan et al. (2016) revealed approximately 75% of animals ADV positive by PCR in some areas.

In Europe (Obon et al., 2011; Wibbelt et al., 2017) and North America (Himsworth et al., 2013), there have been single pox infection reports in Eurasian and North American red squirrels (*Tamiasciurus hudsonicus*) respectively. All three were due to orthopox-like infections and proven to be genetically distinct from those seen in the UK. The Eastern grey squirrel in North America contracts a fibromatous pox infection (Terrell et al., 2002) again, distinct from the UK type. Thus, cross-referencing to other pox virus infections would be an unlikely occurrence in the UK, as since the first reported case (Scott et al., 1981), confirmations are by electron microscopy viral particle detection from skin lesion material. The possibility this scenario will occur in other geographic locations is warranted however, and should be viewed in a local capacity and such additional validation should be undertaken locally.

Red squirrels showed variable ADV and SQPV prevalence, revealing that of the 51 carcasses collected from areas with historically documented ADV cases, 39% gave detectable ADV levels in whiskers using the ADV qPCR. With the nested qPCRs, six additional SQPV positive cases gave 100% SQPV and 50% ADV positives using 30 hairs/sample. The small number of qPCR ADV (9%) detections from the 35 ADV spleen negative animals compared to the 79% ADV positive level detec-

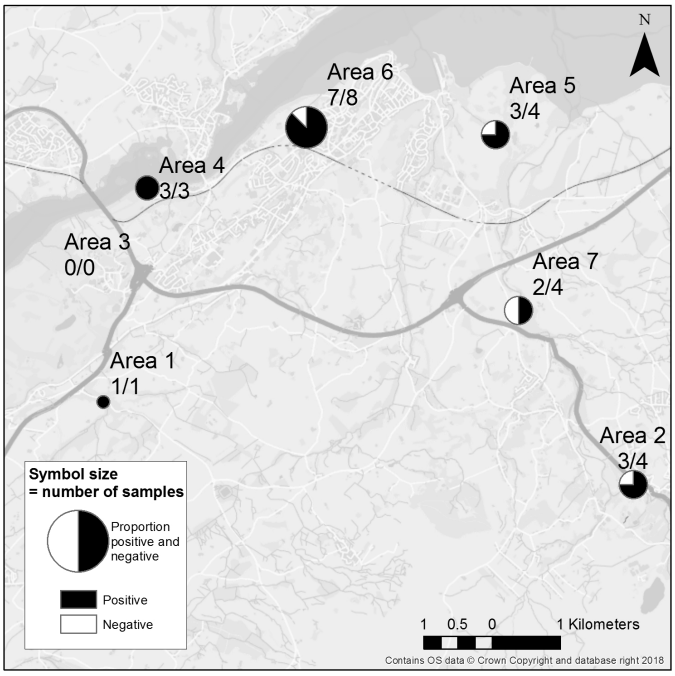

Figure 7 – Locations for ADV nested qPCR hair results from 24 selected spleen and blood negative grey squirrels (Rhyd Ddu and Zoo locations not displayed but data included).

ted with the nested qPCR (Fig. 7) provides evidence that nested qPCR assays may offer sensitivity enhancement, and able to detect lower viral nucleic acid levels in the matrix, providing for higher detection numbers as an obvious benefit.

Using only 80 animals as a study population to validate this new platform may seem limited in its scope, but we were constrained by time and resource availability. However, we can now better determine whether this assay platform's suitability as a surveillance platform has merit in its intended form. In the context of climatic change effects, animal movements, conservation translocations and the spread of non-native species (Gavier-Widén et al., 2012) the need for evolving methods of disease monitoring in mammal populations must be a high priority (Ryser-Degiorgis, 2013). We see our research findings as an important contribution. Adopting a non-invasive hair analysis based surveillance system, would significantly expand the platform's viral surveillance application, allowing carcass analyses where no internal organs are available for examination, (e.g. road casualties and animals recovered from nest boxes). In addition, a potential monitoring tool for zoological collection use to detect potential viral presence prior to captive breeding trans-locations may be of interest.

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Associate Editor: N. Ferrari

Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Supplemental Table S1 Cause of death for 33 of 51 submitted red squirrels.

Supplemental Table S2 Details for primer and probe selections for the PCRs.

Supplemental Table S3 Potential intra-specific and inter-specific infections that may be contracted through predation or at point food sources.